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(21) International Application Number: PCT/US99/20715 (22) International Filing Date: 10 September 1999 (10.09.99) (30) Priority Data: 60/099,793 10 September 1998 (10.09.98) US (71) Applicant (for all designated States except US): PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 800 Capital Square, 400 Locust Street, Des Moines, IA 50319 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ALBERTSEN, Marc, C. [US/US]; 10197 N.W. 74th Avenue, Grimes, IA 50111 (US). BROOKE, Catherine, D. [US/US]; 6017 Greywood Circle, Johnston, IA 50131 (US). GARNAAT, Carl, W. [US/US]; 401 N.E. Innsbruck Drive, Ankeny, IA 50021 (US). ROTH, Bradley, Allen [US/US]; 7425 Hickory Lane, Urbandale, IA 50322 (US). (74) Agents: SPRUILL, W., Murray et al.; Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234-4009 (US).		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: ECDYSONE RECEPTORS AND METHODS FOR THEIR USE (57) Abstract <p>The field of the invention is inducible gene expression systems wherein expression is controlled by a novel ecdysone receptor or its derivatives. The field particularly relates to the isolation and characterization of nucleic acid and polypeptides for a novel ecdysone receptor. The nucleic acid and polypeptides are useful in novel gene expression systems inducible with ecdysone or derivative receptor agonists.</p>		

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ECDYSONE RECEPTORS AND METHODS FOR THEIR USE

FIELD OF THE INVENTION

The field of the invention is inducible gene expression systems wherein expression is controlled by a novel ecdysone receptor or its derivatives. The field particularly relates to the isolation and characterization of nucleic acid and polypeptides for a novel ecdysone receptor. The nucleic acid and polypeptides are useful in novel gene expression systems inducible with ecdysone or derivative receptor agonists.

BACKGROUND OF THE INVENTION

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The steroid hormone 20-hydroxyecdysone, also known as β -ecdysone, controls timing of development in many insects. Ecdysone triggers coordinate changes in tissue development that results in metamorphosis. See generally, Kollman (ed.) *Ecdysone: From Chemistry to Mode of Action*, Thieme Medical Pub., NY (1989), which is incorporated herein by reference. The generic term "ecdysone" is frequently used as an abbreviation for 20-hydroxyecdysone.

The Ecdysone receptor (EcR) polypeptide comprises a ligand-binding domain, a DNA-binding domain, and a transactivating domain. The receptor binds to 20-hydroxyecdysone and transactivates gene expression of a target gene in the nucleus.

20

The ligand-binding domain of the receptor polypeptide provides the means by which the 5' regulatory region of the target gene is activated in response to the hormone. Other chemicals, such as the non-steroidal ecdysone agonist RH5849 (Wing, *Science* 241:467-469 (1988)), will also act as a chemical ligand for the ligand-binding domain of EcR.

25

The DNA-binding domain comprises a sequence of amino acids that binds noncovalently to a response element, a specific nucleotide sequence in a target gene. A response element is located in the 5' regulatory region of a target gene that is activated by the hormone.

The transactivation domain comprises one or more amino acid sequences acting as subdomains to affect the operation of transcription factors during pre-initiation and assembly at the TATA box. The effect of the transactivation domain is to allow repeated transcription initiation events leading to greater levels of gene expression from the target gene. Binding of the ligand causes a conformational change in the receptor polypeptide and allows the transactivation domain to affect transcription of the coding sequence in the target gene, resulting in production of the target polypeptide.

The EcR in *Drosophila* exists in three isoforms, each with an independent biological function. All share the same heterodimeric partner, Ultraspiracle (USP). USP is most homologous to the retinoic acid receptor α , also capable of forming heterodimers with EcR (Thomas *et al.* *Nature* 362:471-475 (1993)). While each of the isoforms independently interacts with ecdysone, the addition of USP greatly enhances the affinity of the complex for binding to ecdysone response elements (EcRE) found in the promoters of genes. Ultraspiracle has been isolated and cloned and its ligand-binding domain identified (Henrich *et al.*, *Nucleic Acids Research* 18:4143-4148 (1990)). See WO 94/01558.

Without bound ligand, the EcR/USP heteroduplex interacts weakly with the EcRE, inhibiting transcription. Binding of ecdysone or other ligand by the EcR/USP complex enhances its affinity for the EcRE, with the conformational changes in the complex functioning to facilitate transcription. The ecdysone heteromeric receptor complex binds the DNA with a high affinity and acts as a potent promoter of DNA transcriptional activation, with transient transcriptional inducibility facilitated by the presence of the ecdysone ligand.

It is often desirable to control the onset and extent of gene expression. The ability to selectively induce the expression of specific genes allows for the manipulation of development and function not only at the cellular but also at the system and organismal level. Thus, ecdysone receptor/Ultraspiracle provides a means of regulating gene expression. It provides for regulation using a chemical that may be extrinsically applied in order to trigger gene activation.

U.S. Patent No. 4,981,784 discusses novel retinoic acid receptors encoding chimeric receptor proteins. The chimeras are constructed by exchanging functional domains between glucocorticoid, mineral corticoid, estrogen-related, thyroid, and

retinoic acid receptors.

U.S. Patent No. 5,310,662 discusses hormone and hormone-like receptors in which the transactivation domains are modified in terms of position or copy number, the receptors having increased transactivation properties.

5 U.S. Patent No. 4,833,080 is directed to the regulation of eukaryotic gene expression controlled by prokaryotic peptides recognizing specific sequences in the gene and either activating or repressing transcription.

U.S. Patent No. 5,554,510 is directed to regulating gene expression by providing a pair of unlike proteins, with unlike DNA-binding domains, which will
10 form a heterodimer, bind to asymmetric DNA binding sites, and depress or increase gene expression.

U.S. Patent No. 5,217,867 is directed to hormone and hormone-like receptors in which changes in position or copy number of the transactivation domain provides increased transactivation.

15 U.S. Patent No. 5,571,696 is directed to members of the steroid/thyroid superfamily of receptors, DNA encoding these receptors, and methods for expressing them in host cells.

WO 90/11273 is directed to steroid/thyroid hormone receptor DNA-binding domain compositions that determine target site specificity, and methods for
20 converting the target site specificity of one receptor into the target site specificity of another.

U.S. Patent No. 5,298,429 is directed to bioassays for determining whether a compound is a hormone receptor agonist or antagonist, the assay involving a DNA sequence encoding a hormone response element operatively linked to a
25 reporter gene.

U.S. Patent No. 5,071,773 discusses hormone receptor bioassays useful to determine whether a protein, suspected of being a hormone receptor, has transcription-activating properties, and evaluating whether compounds are functional ligands for receptor proteins.

30 WO 93/21334 relates to chemically-inducible plant gene expression cassettes in plant cells transformed with the cassettes. An inducible promoter is operatively linked to a target gene, the inducible promoter being activated by a regulator protein also expressed in the cell. In the presence of an effective

exogenous inducer, the activator protein induces expression of the target gene.

WO 93/23431 is directed to mutant steroid hormone receptors and methods for using these as a molecular switch in gene therapy.

WO 94/01558 is directed to various members of the steroid/thyroid
5 superfamily of receptors that can interact with the insect-derived Ultraspiracle receptor to form multimeric species.

WO 92/16546 is directed to DNA segments that are response elements for ligands for several members of the steroid/thyroid superfamily of receptors. Response elements in combination with a functional promoter and gene provide
10 recombinant vectors containing a gene responsive to ligands for members of the steroid/thyroid superfamily of receptors.

WO 93/11235 is directed to the interaction of various members of the steroid/thyroid superfamily of receptors to form multimers of more than one receptor. This modulates the ability of a first receptor to transactivate the
15 transcription of genes maintained under the hormone expression control in the presence of the cognate ligand for the first receptor.

U.S. Patent No. 5,534,418 is directed to methods for controlled production of recombinant proteins in cells. Transcription of the gene encoding the protein is maintained under the control of a transcription control element that is activated by
20 a ligand receptor complex. The complex is formed when a ligand (a hormone or analog of a hormone) is complexed with a receptor (which is a hormone receptor or functional analog).

WO 91/13167 is directed to the isolation of insect DNA sequences having the characteristics of insect steroid receptors.

25 WO 96/27673 discusses recombinant inducible gene expression systems involving the ecdysone receptor. Chimeric receptors are made in which either the ligand-binding domain, the transactivator domain, or the DNA-binding domain are heterologous in the recombinant receptor. The construct is used to activate a target gene in a plant cell.

30 U.S. Patent No. 5,514,578 discloses polynucleotide sequences that encode ecdysone receptors and their expression in host cells.

WO 96/37609 is directed to an ecdysone receptor protein capable of acting as a gene switch responsive to a chemical inducer, enabling external control of a

gene.

WO 93/03162 is directed to a method for inducing gene expression by contacting an ecdysteroid with an ecdysteroid receptor polypeptide in a mammalian cell. The cell contains a DNA binding sequence for the receptor.

5 U.S. Patent No. 5,880,333 is directed to a method for controlling gene expression in a plant through the expression of class II steroid and thyroid hormone superfamily of nuclear hormones. Chimeric receptors are generated in which either the ligand-binding domain, the DNA binding domain or the transactivator domain are heterologous in the recombinant receptor. The chimeric receptors are
10 used to regulate expression of target sequences.

U. S. Patent No. 5,432,068 is directed to a method for controllably rendering plants male fertile by using an inducible promoter to regulate expression of a gene critical to male fertilization such that when the gene is "off," the plant is sterile; however, when the promoter is induced, the plant becomes fertile. In
15 particular, it is directed to a method whereby a gene affecting flavanol production in a plant is controlled in a manner rendering the plant conditionally male fertile. Related U. S. Patent No. 5,478,369 discloses nucleotide and amino acid sequences mediating male fertility in plants.

U. S. Patent Nos. 5,824,524 and 5,850,014 also relate to control of plant
20 fertility by providing a constitutively sterile plant, wherein fertility may be induced using male fertility MS45 DNA molecules or genes impacting flavonone production.

U. S. Patent No. 5,859,341 is directed to a method for providing heritable, externally controllable male fertility through selective induction of
25 microsporogenesis.

While some ecdysone receptors are known, there is a need for the isolation and use, especially in plants, of additional receptors. The invention is thus directed to novel ecdysone receptors. While the invention may have an impact on the furtherance of the understanding of insect physiology, the potential use of the
30 receptor peptides in the control of gene expression, particularly in plants, is of primary interest.

SUMMARY OF THE INVENTION

Accordingly, the invention is directed to a novel protein from the insect genus *Pyrilidae*, the protein being responsive to ecdysone.

5 The invention is particularly directed to the protein from the species *Ostrinia*, and especially *Ostrinia nubilalis*.

The invention encompasses a novel protein that acts as an ecdysone receptor in these species.

10 The invention is specifically directed to a protein comprising the amino acid sequence in SEQ ID NO 2.

15 The invention also encompasses a further novel protein from a *Pyrilidae* species that binds with the novel ecdysone receptor to form a heterodimer. This protein can be designated the "Ultraspiracle" protein. Thus the invention specifically is directed to a protein comprising the amino acid sequence in SEQ ID NO 3.

The proteins are useful, alone or in combination, for activating a target gene in response to a chemical inducer such as ecdysone, an ecdysone derivative, or an ecdysone mimic.

20 The invention is also directed to a chimeric receptor protein in which one or more of the ligand binding, DNA binding, or transactivation domains in the novel ecdysone receptor are obtained from a source heterologous with respect to the other domains present in the chimeric receptor protein. Chimeric receptor proteins provide flexibility in the choice of ligand and choice of target sequence to be activated, and thus allow controlled gene expression in a cell or in an organism.

25 The choice of the heterologous source will depend upon the desired specificity and effectiveness level for transactivation, ligand binding, or DNA binding. Accordingly, the chimeric receptor molecules confer the advantage of: (1) maximizing transactivation by using a transactivator domain stronger than the native domain and allowing transactivation in a specific host cell by using a
30 transactivator that is compatible with a particular host cell; (2) providing induction by a selected chemical ligand by replacing the ligand binding site with a binding site for another ligand; and (3) use of specific response elements by replacing the native DNA binding site with a site that recognizes different response elements

(i.e. other than EcRE).

The invention is also directed to an isolated nucleotide sequence encoding these proteins. In particular, the invention is directed to the nucleotide sequence shown in SEQ ID NO: 1 (ecdysone receptor) and SEQ ID NO: 3 (Ultraspiracle).

5 The invention also encompasses expression vectors comprising the nucleotide sequences.

The invention also encompasses cells containing the expression vectors and/or expressing the nucleotide sequences.

10 The invention also provides methods for screening for ligands that bind to the proteins described herein.

In preferred embodiments of the invention, the cell in which the receptor is expressed and the target gene activated is a plant cell.

The invention also provides methods to regulate plant fertility.

15

DETAILED DESCRIPTION OF THE INVENTION

20 The invention is directed to novel proteins that are responsive to ecdysone, being derived from the genus *Pyrilidae* and particularly from the species *Ostrinia*, specifically *Ostrinia nubilalis*. Compositions of the invention include a novel Ecdysone receptor (EcR) and the Ultraspiracle (USP) receptor. Both of these receptors have homology to members of the steroid/hormone receptor superfamily and are involved in activating transcription of a target gene in response to a chemical inducer. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid

25 sequences shown in SEQ ID NOS: 2 or 4. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example those set forth in SEQ ID NOS: 1 or 3 and fragments and variants thereof.

30 The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free

of chemical precursors or other chemicals when chemically synthesized.

Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

It is understood, however, that there are embodiments in which preparations that do not contain the substantially pure protein may also be useful. Thus, less pure preparations can be useful where the contaminating material does not interfere with the specific desired use of the peptide.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain one or more of the functional biological activities of the native protein. Accordingly, a fragment of a nucleotide sequence may retain a functional ligand-binding domain, transactivation domain or DNA binding domain.

A fragment of an Ultraspiracle or Ecdysone receptor nucleotide sequence that encodes a biologically active portion of an Ultraspiracle or Ecdysone receptor of the invention will encode at least 15, 25, 30, 50, 100, 150, 200, 250, 300, 350, 400, 450 contiguous amino acids, or up to the total number of amino acids present in a full-length receptor of the invention (for example, 546 or 460 amino acids for SEQ ID NOS: 2 and 4, respectively). Fragments of a nucleotide sequence encoding an Ultraspiracle or Ecdysone receptor that are useful as hybridization

probes for PCR primers generally need not encode a biologically active portion of an Ultraspiracle or Ecdysone receptor.

Thus, a fragment of a nucleotide sequence encoding an Ultraspiracle or Ecdysone receptor may encode a biologically active portion of a receptor, or it may
5 be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an Ultraspiracle or Ecdysone receptor can be prepared by isolating a portion of one of the nucleotide sequences encoding a receptor of the invention, expressing the encoded portion of the receptor (e.g., by recombinant expression *in vitro*), and assessing the activity of
10 the encoded portion of the receptor. Nucleic acid molecules that are fragments of an Ultraspiracle or Ecdysone receptor nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, or 1,400, 1,500, 1,600, 1,700, 1,800 nucleotides, or up to the number of nucleotides present in a full-length Ultraspiracle or Ecdysone
15 receptor nucleotide sequence disclosed herein (for example, 2,126 and 1,837 nucleotides for SEQ ID NOS: 1 and 2, respectively).

Also encompassed in these fragments are nonfunctional fragments that are useful, among other things, for diagnostic assays. Accordingly, a nonfunctional ligand binding domain fragment can have use as a binding antagonist that will
20 compete with a functional ligand binding domain. Such a fragment can, for example, bind a ligand but be unable to confer the conformational change necessary for DNA binding. Similarly, a nonfunctional DNA binding domain may bind to DNA in such a manner that it prevents transactivation and consequent gene expression, while also preventing DNA binding by a functional DNA binding
25 domain. Similarly, a nonfunctional transactivation domain may interfere with transcription induced by host cell components.

Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at
30 least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the proteins of the invention.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the

degeneracy of the genetic code, encode the amino acid sequence of one of the Ultraspiracle or Ecdysone receptor polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain
5 reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a Ecdysone or Ultraspiracle receptor of the invention. Generally, nucleotide sequence variants of the invention will have at least 40%, 50%, 60%, 70%, generally, 80%,
10 preferably 85%, 90%, up to 95%, 98% sequence identity to its respective native nucleotide sequence.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or
15 more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or from human manipulation.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such
20 manipulations are generally known in the art. For example, amino acid sequence variants of the Ultraspiracle or Ecdysone receptor proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-
25 382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl.
30 Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to retain a functional ligand-binding domain, transactivation domain or DNA binding domain. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by the ability of the receptor to bind ligand, interact with DNA, or activate transcription.

Variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different Ultraspiracle or Ecdysone receptor coding sequences can be manipulated to create a new receptor possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the Ecdysone or Ultraspirical receptor genes of the invention and other known steroid/hormone receptors or gene activator proteins to obtain a new gene coding for a protein with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Cramer *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al.* (1997) *Proc. Natl.*

Acad. Sci. USA 94:4504-4509; Crameri *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

In addition, protein variants encompassed by the present invention include those that contain mutations that either enhance or decrease one or more domain
5 functions. For example, in the ligand binding domain, a mutation may be introduced that increases the sensitivity of the domain to a specific ligand so that gene expression can be increased. Alternatively, it may be desired to introduce a mutation that reduces sensitivity of the domain to an undesirable ligand that may compete with a desired ligand. Similarly, mutations may be introduced into the
10 DNA binding and transactivation domains that increase or reduce the functionality of these domains. As an alternative to the introduction of mutations, increase in function may be provided by increasing the copy number of these domains. Thus, the invention also encompasses proteins in which one or more of the domains is provided in more than one copy.

15 The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire receptor sequences set forth herein or
20 to fragments thereof are encompassed by the present invention.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any organism of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.*
25 (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New
30 York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P , or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the receptor sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire sequence of Ultraspiracle or Ecdysone receptor disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding Ultraspiracle or Ecdysone receptor sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among Ultraspiracle or Ecdysone receptor sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding Ecdysone receptor or Ultraspiral sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in

different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m)

for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Thus, isolated sequences that encode for an Ecdysone receptor or Ultraspical protein and which hybridize to the Ecdysone receptor or Ultraspical sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least 40% to 50% homologous, about 60% to 70% homologous, and even about 80%, 85%, 90%, 95% to 98% homologous or more with the disclosed sequences. That is, the sequence similarity of sequences may range, sharing at least about 40% to 50%, about 60% to 70%, and even about 80%, 85%, 90%, 95% to 98% sequence similarity.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the

entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; by the homology alignment algorithm of Needleman *et al.* (1970) *J. Mol. Biol.* 48:443; by the search for similarity method of Pearson *et al.* (1988) *Proc. Natl. Acad. Sci.* 85:2444; by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California; GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90; Huang *et al.* (1992) *Computer Applications in the Biosciences* 8:155-65, and Person *et al.* (1994) *Meth. Mol. Biol.* 24:307-331; preferred computer alignment methods also include the BLASTP, BLASTN, and BLASTX algorithms (see Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410). Alignment is also often performed by inspection and manual alignment. Sequence alignments are performed using the default parameters of the above mentioned programs.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a

specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of

proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like.

Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and
5 most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However,
10 stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the
15 maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates
20 that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman *et al.* (1970) *J. Mol. Biol.* 48:443.
25 An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue
30 positions that are not identical may differ by conservative amino acid changes.

The protein can be purified from cells that naturally express it, purified from cells that have been altered to express it (i. e. recombinant) or synthesized using protein synthesis techniques that are well known in the art. In a preferred

embodiment, the protein is produced by recombinant DNA methods. In such methods a nucleic acid molecule encoding the protein is cloned into an expression vector as described more fully herein and expressed in an appropriate host cell according to known methods in the art. The protein is then isolated from cells
5 using protein purification techniques well known to those of ordinary skill in the art. Alternatively, the protein or fragment can be synthesized using peptide synthesis methods well known to those of ordinary skill in the art.

The invention also encompasses chimeric proteins in which a heterologous protein, having an amino acid sequence that is not substantially homologous to the
10 protein of the invention, forms a fusion protein with a protein, or fragment or variant thereof, of the invention. These proteins may or may not be operatively linked. An example of operative linkage is fusion in-frame so that a single protein is produced upon translation. Such fusion proteins can, for example, facilitate the purification of a recombinant protein. In another embodiment, the fusion protein
15 may contain a heterologous signal sequence at the N-terminus facilitating its secretion from specific host cells. The expression and secretion of the protein can thereby be increased by use of the heterologous signal sequence.

The invention is particularly directed to proteins in which one or more domains in the protein described herein are operatively linked to heterologous
20 domains having homologous functions. Thus, the ligand binding domain can be replaced with a ligand binding domain for other ligands. Thereby, the control of gene expression is based on a ligand other than ecdysone but gene expression in the nucleus depends upon ecdysone response elements. Alternatively, the native ligand binding domain may be retained while the DNA binding domain is replaced
25 with a binding domain that recognizes a heterologous response element. Thus, while the sequence of events for gene expression is initiated by ecdysone or other appropriate ligand, ultimate gene expression depends upon the presence of a response element corresponding to the heterologous DNA binding sequence. Furthermore, the transactivation region can be replaced depending upon the cell
30 type in which gene expression is desired. Thus, in order to recruit transcription factors in a specific cell type, a transactivation region compatible with that cell type can replace the native transactivation domain.

Ligand binding domains may be obtained from members of the class II receptor

proteins of the steroid/thyroid hormone superfamily of nuclear receptors, which include, but are not limited to, retinoic acid receptor, vitamin D receptor, and thyroid hormone receptor.

Transactivation domains may also be obtained from members of the class II
5 receptor proteins of the steroid/thyroid hormone superfamily of nuclear receptors, which include, but are not limited to, retinoic acid receptor, vitamin D receptor, and thyroid hormone receptor. Alternatively, the transactivation domain may be obtained from other transcriptional activators including, but not limited to VP16 from herpes simplex, maize C1, and GAL4.

10 DNA binding domains may also be obtained from members of the class II receptor proteins of the steroid/thyroid hormone superfamily of nuclear receptors, which include, but are not limited to, retinoic acid receptor, vitamin D receptor, and thyroid hormone receptor. Alternatively, the DNA binding domain may be obtained from other transcriptional activators including, but not limited to
15 LexA, and GAL4.

As used herein, the term "recombinant" refers to *in vitro* cleavage and religation of nucleotide sequences as well as cleavage and ligation in the cell or in the organism that results from, for example, integration of exogenous sequences into the cellular endogenous sequences, as by homologous recombination.

20 The nucleic acid can be purified from cells that naturally express it, for example, a cell in which the sequence is amplified, or from cells in which it has been exogenously introduced, i.e., a recombinant cell. Alternatively, nucleic acid can be synthesized using synthesis techniques well known in the art. Thus, nucleotides encoding the proteins described herein can be cloned into expression
25 vectors, amplified, and expressed in an appropriate host cell.

The invention also encompasses nucleotide sequences that provide fusion proteins in which a heterologous protein, having an amino acid sequence not substantially homologous to the proteins of the invention, forms a fusion protein with the proteins of the invention. These nucleotide sequences may or may not
30 provide for operative linkage.

The invention is particularly directed to nucleotide sequences encoding proteins in which one or more domains in the protein is operatively linked to a heterologous domain having a homologous function. Thus, the ligand binding

domain can be replaced with a ligand binding domain for another ligand. Alternatively, the native ligand binding domain can be retained while the DNA binding domain is replaced with a binding domain recognizing a heterologous response element. Finally, the transactivation region can also be replaced by a
5 desired transactivation region.

The invention further encompasses cells containing, in addition to the receptor expression vectors (i.e. the ecdysone receptor, Ultraspiracle, or chimeric derivatives), nucleotide sequences that serve as a target for proteins expressed from the receptor expression vectors. The target comprises a regulatory region operably
10 linked to a nucleotide sequence encoding a target polypeptide whose expression is controlled by addition of a ligand and consequent activation of the receptor protein. The regulatory region contains a core promoter sequence, a sequence that allows the initiation of transcription, and response elements for binding receptor.

Accordingly, the expression of any desired coding sequence can be
15 desirably controlled as long as the promoter controlling the transcription of the coding sequence is designed to contain a response element complementary to the DNA binding domain of the receptor polynucleotide. The level of expression can be controlled by the choice of transactivator. The induction of the expression can be controlled by the ligand choice.

20 Accordingly, the invention is further directed to methods for controlling the expression of a specific gene and the production of a gene product by providing a chemical ligand that interacts with the proteins described herein.

Either the ecdysone receptor or the USP protein or a combination thereof (as a heterodimer) can be used. In embodiments in which the ecdysone receptor is
25 used alone, one or more mutations may be made in one or more of the domains in order to achieve a desired level of gene activation.

The system can consist of two or more expression cassettes used to transform a host cell. The first expression cassette could contain the sequences for the EcR and/or USP proteins or their equivalents. Transcription of these genes can
30 be placed under the control of a promoter, such as a constitutive or inducible promoter or a tissue - or cell cycle-preferred promoter. The second expression cassette contains a target sequence.

The target sequence encodes a protein of interest for which ligand-

inducible expression is desired. Such target sequences could include, but are not limited to, genes encoding insecticide or herbicide resistance, nutritional factors, growth inducers, and genes required for fertility. Such a target sequence follows an ecdysone or other appropriate response element contained within its promoter region.

In one embodiment, the EcR/USP heterodimer expressed in the transformed cell preferentially binds the ecdysone response element upstream of the target sequence. When the ligand for the heterodimer is introduced into the host cell the EcR/USP/ligand complex acts to facilitate gene expression. Specific recognition sequences comprising a ligand response element for EcR/USP recognition are well-known in the art and may be effectively engineered into the promoter region of a target gene through commonly employed molecular biological techniques. See, for example, Crispi, S., *J. Mol. Biol.* 275(4):561-574 (1998); Antoniewski, C., *Mol. Cell. Biol.* (6):2977-2986 (1996); Jones, C., *Insect Biochem. Mol. Biol.* (9): 875-882 (1994); Antoniewski, C., *Mol. Gen. Genet.* 249(5):545-556 (1995 Dec 15); Antoniewski, C., *Mol. Cell. Biol.* (7):4465-4474 (1994); Antoniewski, C., *Insect Biochem. Mol. Biol.* (1):105-114 (1993).

A preferred embodiment of this invention thus embodies the response element recognized by either the EcR/USP complex or a modified derivative thereof, including EcR or USP alone in a chimeric construct, which is engineered into the promoter of a target gene. This construct may then be employed to co-transform a host cell along with an expression cassette containing the EcR and/or USP nucleic acid sequences or a derivative thereof. In the presence of the ligand recognized by the EcR receptor polypeptide, the target gene is selectively induced. Induction in this fashion may be ligand concentration dependent, with increasing concentrations of the ligand resulting in higher levels of gene expression. Thus, by linking a ligand responsive element to a peptide encoding segment, a protein expression system may be developed in which protein production is regulated by the administration of a ligand.

In another embodiment, expression of EcR alone or a chimeric derivative thereof, regulates transcription of the target sequences. In this embodiment a response element recognized by EcR or a chimeric derivative thereof is engineered into the transcriptional regulatory region of the target sequence. Addition of

ligand, results in the activation of EcR and the expression of the target sequences.

The term "nucleic acid" refers to all forms of DNA such as cDNA or genomic DNA and RNA such as mRNA, as well as analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecules can be single
5 stranded or double stranded. Strands can include the coding or non-coding strand.

The proteins or other component polypeptides described herein may be used alone or in combination with other proteins or agents to facilitate gene expression. It is recognized in the art that the components of the ecdysonal binding system may be substituted with other components of related hormone response
10 elements to achieve substantially similar effects.

The nucleotide sequences encoding the proteins of the invention can be manipulated and used to express the protein in a variety of hosts including microorganisms and plants. It is recognized that the proteins and DNA sequences of the invention may be used alone or in combination with other proteins.

15 The proteins of the invention can be used in expression cassettes for expression in any host of interest. Such expression cassettes will comprise a transcriptional initiation region linked to the gene encoding the protein of interest. Such an expression cassette can be provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the
20 regulatory regions. The expression cassette may additionally contain selectable marker genes suitable for the particular host organism to be used.

The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is
25 intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

Any promoter or promoter element capable of driving expression of a
30 coding sequence can be utilized. For instance, constitutive promoters include, for example, the core promoter of the Rsyn7 (copending U.S. Application Serial No. 08/661,601); the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin

(Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Application Serial No. 08/409,297), and the like. Other
 5 constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

Alternatively, tissue-preferred promoters can be utilized to target enhanced EcR or USP expression within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997)
 10 *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results*
 15 *Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Of particular interest are promoters which direct expression in the
 20 reproductive organs of the plant. Such promoters include, but are not limited to, for example, anther, tapetal, pistil, and ovule-preferred promoters. Examples of anther-preferred promoters include ant32 and ant43D (U.S. Patent Application Serial No. 07/908,242), anther (tapetal) promoter B6 (Huffman *et al.* (1993) *J. Cell. Biochem.* 17B:Abstract #D209) and promoter 5126 (U. S. Patent No.
 25 5,795,753). Other examples of anther-preferred promoters may also be found in U.S. Patent Application 5,470,359. A pistil-preferred promoter, includes for example, the modified S14 promoter (Dzelkalns *et al.* (1993) *Plant Cell* 5:855.

The transcriptional cassette will include in the 5' -3' direction, the orientation of transcription, a transcriptional initiation region, a translational
 30 initiation region, a DNA sequence of interest, a translational termination region, and a transcriptional termination region functional in plants. The termination region may be native with respect to the transcriptional initiation region, may be native with respect to the DNA sequence of interest, or may be derived from other

sources. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau *et al.*, (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; 5 Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* 1989) *Nucleic Acids Res.* 17:7891-7903; Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639.

Expression cassettes for the expression of co-transformed target genes will include a promoter region specific for response element binding in a position 10 proper to promote expression of the target gene. Thus such a region will be included a proper distance upstream of the cloning site for the gene and in the proper orientation. Otherwise, expression cassettes for target genes may include all of the required elements previously discussed.

The nucleotide sequences encoding the proteins or polypeptides of the 15 invention are particularly useful in the genetic manipulation of plants. In this manner, the genes of the invention are placed into expression cassettes for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to the gene of interest. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. 20 Alternatively, other gene(s) of interest can be provided on other expression cassettes. Where appropriate, the gene(s) may be otherwise optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant preferred codons for improved expression. Methods are available in the art for synthesizing plant preferred genes. See, for example, U.S. 25 Patent Nos. 5,380,831, 5,436, 391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, 30 and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin

secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translational leaders are known in the art and include: picornavirus
5 leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T.R., and Moss, B. (1989) *PNAS USA*, 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology*, 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak, D.G., and P.
10 Sarnow (1991) *Nature*, 353:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S.A., and Gehrke, L., (1987) *Nature*, 325:622-625; tobacco mosaic virus leader (TMV), (Gallie, D.R. *et al.* (1989) *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. *et al.* (1991) *Virology*, 81:382-385). See
15 also, Della-Cioppa *et al.* (1987) *Plant Physiology*, 84:965-968. Other methods known to enhance expression can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and,
20 as appropriate, in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resection, ligation, PCR, or the like may be
25 employed, where insertions, deletions or substitutions, *e.g.* transitions and transversions, may be involved.

The invention encompasses various ligands. The first non-steroidal ecdysteroid agonists, dibenzoyl hydrazines, typified by RH-5849 [1,2-dibenzoyl, 1-tert-butyl hydrazide], which is commercially available as an insecticide from
30 Rohm and Haas, were described back in 1988. Another commercially available compound in this series is RH-5992 [tebufenozide, 3,5-dimethylbenzoic acid 1-1 (1,1-dimethylethyl)-2(4-ethylbenzoyl) hydrazide]. These compounds mimic 20-

hydroxyecdysone (20E) in both *Manduca sexta* and *Drosophila melanogaster*.

- These compounds have the advantage that they have the potential to control insects using ecdysteroid agonists that are non-steroidal. Further examples of such dibenzoyl hydrazines are given in US Patent No. 5,117,057 to Rohm and Haas, and Oikawa *et al.*,
 5 *Pestic. Sci.* 41:139-148 (1994). However, it will be appreciated that any inducer of the gene switch of the present invention, whether steroidal or non-steroidal, and which is currently or becomes available, may be used. A preferred ligand is methoxyfenozide.

Chemical Abstract Name:	Benzoic Acid, 3-methoxy-2-methyl-2-(3,5-dimethylbenzoyl)-2-(1, 1-dimethylethyl) hydrazide
IUPAC Name:	N'-t-butyl-N'-(3,5-dimethylbenzoyl)-3-methoxy-2-methylbenzohydrazide
Common Name:	methoxyfenozide
Trade Name:	INTREPID™ Insecticide
CAS Number:	161050-58-4
Empirical Formula:	C ₂₂ H ₂₈ N ₂ O ₃
Molecular Weight:	368.47

- 10 Analogues of ecdysone, such as *Muristerone A*, are also encompassed by the present invention.

- The peptide products of the nucleotide sequences of the invention may also be used to elucidate other potential ligand analogs. The screening of diverse libraries of small molecules created by combinatorial synthetic methods may be
 15 used to identify compounds with binding affinity. One of skill in the art would be able to use the peptide sequences provided to develop sensitive assays to screen for both agonists and antagonists of ligand binding.

In constructing expression cassettes for the EcR or USP expression as well as for target gene expression, a variety of commonly recognized plasmids may be

employed. One of skill in the art would recognize that competent cells could be transformed with expression cassettes that facilitate autonomous replication or integrate into the host cell chromosome. The EcR, USP and target gene could be included in a single cassette or multiple cassettes. Further it is envisioned by the
5 inventors that the EcR or USP could be employed to regulate the expression of multiple target genes in a transformed host cell.

The EcR or USP peptide of the invention may also be combined with other members of the steroid/hormone receptor family. See, for example, Rowe, A.
10 (1997) *Int. J. Biochem. Cell. Biol.*, (2): 275-278; Glass, C. (1996) *J. Endocrinol.*, 150(3):349-357; Chambon, P. (1996) *FASEB J.*, 10(9): 940-954; Mangelsdorf, D. (1996) *Cell*, 83(6): 841-850. Members of the steroid/thyroid hormone receptor superfamily of DNA binding nuclear hormone receptor have been shown to react across species. Thus the substitution of trans retinoid acid, thyroid hormone,
15 vitamin D or peroxisome proliferator-activated receptors for EcR could facilitate DNA interaction. Such chimeric proteins could provide an important reflection of the binding properties of the members of the superfamily. Such combinations could be further used to extend the range of applicability of these molecules in a wide range of systems or species that might not otherwise be amenable to native or
20 relatively homologous proteins. Thus chimeric constructs could be substituted into systems in which a native construct would not be functional because of species specific constraints. Hybrid constructs may further exhibit desirable or unusual characteristics otherwise unavailable with the combinations of native proteins.

In another embodiment of the invention, methods are provided for
25 screening for ligands that bind to the proteins described herein. Accordingly, both the proteins and relevant fragments thereof (for example, the ligand binding domain) can be used to screen for compounds that bind to the receptor and thus, induce gene expression. The assays may be cell-based or cell free assays which include the steps of exposing a ligand binding domain, either soluble or in
30 association with a cell, to a candidate compound and either detecting the formation of a complex or detecting a biological event associated with gene expression caused by the activated receptor.

In other embodiments of the invention, it may be desirable to negatively

control gene expression, particularly when expression of that gene is no longer desired or if it is desired to reduce that gene expression to a lower level. In this embodiment, expression could be blocked by the use of antisense molecules directed against the gene of interest. For example, see U. S. Patent Nos. 5,728,558
5 and 5,741,684.

In further embodiments of the invention, antibodies are used to detect receptor expression in a cell. In preferred embodiments, detection is in transgenic plants and directed to assessing expression in various parts of the plant. Detection of the protein could be *in situ* by means of *in situ* hybridization of tissue sections
10 but may also be analyzed by bulk protein purification and subsequent analysis by Western blot or immunological assay of a bulk preparation. Alternatively, gene expression can be expressed at the nucleic acid level by techniques well known to those of ordinary skill in any art using complimentary polynucleotides to assess the levels of genomic DNA, mRNA, and the like. As an example, PCR primers
15 complimentary to the nucleic acid of interest can be used to identify the level of expression.

Plants of interest include, but are not limited to corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*),
20 sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea
25 (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), oats, barley, vegetables, ornamentals, and conifers. Preferably plants include corn, soybean, sunflower, safflower, Brassica, wheat, barley, rye, alfalfa, rice and sorghum. It is understood, however, that the invention encompasses
30 embodiments in which host cells are other than plant cells in which a chimeric receptor contains an ecdysone ligand binding domain but heterologous domains derived from other types of cells, for example, invertebrate cells such as insects and fungi and animal cells such as avian and mammalian.

The compositions of the present invention are preferably used to transform a plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like, can be obtained. By "transformed" is intended the stable introduction of DNA into the genome of the cell. Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Pat No. 5,563,055), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney *et al.*, U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed.

Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

It is envisioned by the inventors that the system will be useful for selectively inducing the expression of specific proteins at a specific time in a plant's developmental cycle. A target gene or genes may be introduced that would enhance the nutritional value of a specific crop. The component could be induced just prior to harvest, or low doses of ligand could be administered throughout growth and development to create stores of a specific product. The system could also be used to selectively induce insecticidal properties or herbicide resistance. In the case of herbicide resistance, it is envisioned by the inventors that the ligand could be introduced to plants by spraying prior to the application of herbicides to clear otherwise unwanted competitors. The inventors further envision the use of the system in heightening plant resistance to environmental factors. Proteins could be selectively induced in response to inclement weather conditions to transiently enhance plant resistance to such conditions as cold, drought or soil over saturation.

It is envisioned that the use of tissue-preferred promoters known to those of skill in the art could limit inducibility to specific tissues or cell types. The inventors envision regulating the expression of EcR and/or USP or their chimeric derivatives with tissue-preferred promoters. This combination will activate transcription of target sequences only in specific tissues or at specific

developmental stages. The system could thus be used to selectively induce expression in the seed or in the reproductive structures of a plant with little or no expression in other areas of the organism. Thus products of expression could be compartmentalized in or away from areas that might be the subject of human
5 consumption.

An embodiment of particular interest to the inventors is the use of the claimed invention in altering the fertility or system of reproduction in developing plants. The inventors envision the use of the ecdysone inducible expression system to promote or repress the expression of genes in tissues of plants that facilitate
10 reproduction. One of skill in the art would recognize that specific genes are known that may be selectively induced or expressed in order to regulate the fertility or mode of reproduction of a given plant. For example, U. S. Patent No. 5,432,068 teaches a method for controllably rendering plants male fertile by using an inducible promoter to regulate expression of a gene critical to male fertility such
15 that when the gene is "off," the plant is sterile; however, when the promoter is induced, the plant becomes fertile. Therefore, one embodiment of the present invention is the use of the ecdysone inducible expression system to regulate male fertility in plants using such a method.

Further, to render the reproductive process ineffective, formation of viable
20 zygotes can be prevented. This may be achieved through a variety of means, including for example, any disruption or alteration of a process that is critical to the formation of viable gametes. Male sterility may result from defects leading to either a lack of pollen formation or the production of pollen that is incapable of effective fertilization. Female sterility may result from the disruption of tissues
25 required for the pollen germination, growth or fertilization. This may result from, for example, the failure to produce functional embryo sacs, ovaries, pistils, stigmas, or transmitting tracts. In addition, an alternative mode of reproduction is apomixis in which progenies are produced asexually using female floral components that have not undergone meiosis or normal double fertilization.

30 Target sequences encoding proteins of interest that would be useful in disrupting the fertilization process include, for example, Adenine Phosphoribosyltransferase (APRT) (Moffatt *et al.* (1988) *Plant Physiol.* 86: 1150-1154, ribonuclease from *Bacillus amyloliquefaciens* (Mariani *et al.* (1990) *Nature*

347:737-741), indole acetic acid-lysine synthetase from *Pseudomonas syringae* (Romano et al. (1991) *Genes and Development* 5: 438-446), pectate lyase *pelE* from *Erwinia chrysanthemi* EC16 (Keen et al. (1986) *J. Bacteriology* 168:595), Cyt A toxin from *Bacillus thuringiensis israeliensis* (McLean et al. (1987) *J. Bacteriology* 169: 1017-1023), T-urf13 from cms-T maize mitochondrial genomes (Braun et al. (1990) *Plant Cell* 2:153, *gin* recombinase from phage Mu (Maeser et al. (1991) *Mol. Gen. Genet.* 230:170-176), and diphtheria toxin A-*chanin* (Greenfield et al. (1983) *Proc. Natl. Acad. Sci.* 80: 6853)

The inventors envision the transformation of plants with a target sequence encoding a protein of interest that disrupts the fertilization process. The use of anther and pistil preferred promoter to control expression of *EcR* and/or *USP* or their chimeric derivative ensures the expression of the target sequences in tissues that are necessary for fertility. The target sequences will therefore not be deleterious to other plant tissues.

For example, transgenic plants and plant cells may be transformed to contain multiple DNA constructs. The first DNA construct encodes an Ecdysone receptor or a chimeric derivative thereof. This DNA construct comprises an anther specific or preferred specific promoter, an organism specific transcription activator, a DNA binding domain, and an *EcR* ligand-binding domain. The second DNA construct comprises an appropriate transcriptional response element operably linked to a constitutive promoter operably linked to a target sequence. Treatment of the transgenic plant with ecdysone would lead to expression of the target sequence and render the plant infertile.

In another embodiment, a third DNA construct is introduced into the plant, and comprises a tissue-preferred promoter operably linked to a *USP* coding sequence or a chimeric derivative thereof. In this embodiment, following treatment with ecdysone, expression of the DNA construct encoding the target sequence is regulated by the *USP/EcR* heterodimer.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1: Isolation of Ecdysone Receptor and Ultraspiracle

For library construction, total RNA was isolated from the European corn borer, *Ostrinia nubilalis*. Corn borer larvae (for example, a mix of stage 2, 3, and 4, equal weight) can be pulverized in liquid nitrogen, homogenized, and total RNA extracted by standard procedures. PolyA RNA was isolated from the total RNA using the PolyATact system from Promega Corporation, Madison, WI. cDNA synthesis was performed and unidirectional cDNA libraries were constructed according to the ZAP Express cDNA synthesis kit from Stratagene, La Jolla, CA. cDNA was amplified by PCR, sized and properly digested with restriction fragments to be ligated into a vector. Probes for the EcR and USP genes were isolated from the cDNA. The cDNA and the degenerate primers were combined in a PCR mix. After amplification, prominent bands were subcloned and sequenced and the fragments obtained can be used to probe for EcR or USP from the corn borer library. The primers used to amplify EcR were 5' CGG AAT TCG GNT AYC AYT AYA AYG C 3' (SEQ ID NO: 5), and 5' CCT CGG GAC SAC TTC GTG AAT CC 3' (SEQ ID NO: 6). For USP the following primers were used. 5' TGT GAR GGC TGC AAR GGS TTC TT 3' (SEQ ID NO: 7), and 5' GCC AGM GCG AGC AGC AG 3' (SEQ ID NO: 8). Subcloned cDNA was sequenced to identify sequences with the proper peptide identity corresponding to published sequences. These fragments were used to probe a cDNA library from *Ostrinia nubilalis* and a full length coding sequence was obtained. The coding sequence was ligated into an expression cassette and sequenced

Example 2:Transactivation of a Reporter Sequence by the Ecdysone and USP Receptors
using Transient Expression Assays

To examine the effect of the Ecdysone and USP receptors on promoter-driven expression, a transient transactivation assay was developed using a cell suspension culture derived from maize embryos. (See also, U.S. Patent No. 5,886,244.) A reporter plasmid, designated PHP11545, was constructed in the pSP72 vector and contained 8 LexA operators, a minimal fragment of the CaMV-

59 constitutive promoter, the Omega' leader sequence, Adh intron 1, the coding sequence for firefly luciferase, and the PinII terminator sequences.

Two DNA constructs encoding effector proteins were generated. A chimeric Ecdysone receptor construct designated PHP10956 was generated.

- 5 PHP10956 contains the Nopaline synthase (NOS) promoter, the Omega' leader sequence, Adh1 intron, and the potato protease inhibitor II (PinII) termination sequences cloned into a pUC vector. A fragment encoding a fusion between the maize C1 transactivation domain, the LexA DNA binding domain, and the ligand-binding domain of the Ecdysone receptor was cloned downstream of the intron.

- 10 A DNA construction containing the USP coding sequences was generated and designated PHP10967. PHP10967 contains the Nos promoter, the Omega' leader sequence, Adh1 intron, and the PinII terminator sequences cloned into the pUC vector. The nucleic acid sequence encoding the USP coding region was inserted downstream of the intron.

- 15 These constructs were introduced into embryogenic suspension cells by microprojectile bombardment as described by Unger *et al.* (1993) *The Plant Cell* 5:831-841. Plasmid DNA encoding effector and reporter sequences was mixed with 50 μ l (15 mg/ml) of 1.0 μ m tungsten microprojectiles. The concentration of the plasmids encoding the effector and reporter sequences is shown in Table 1.

- 20 DNA was precipitated by addition of 50 μ l 2.5 M CaCl_2 and 20 μ l 0.1M spermidine. Microprojectiles and precipitated DNA were centrifuged to a pellet and the supernatant was removed. The pellet was washed by sonication in the presence of 250 μ l absolute ethanol, centrifuged and ethanol removed. Sixty μ l of fresh absolute ethanol was added and this mixture sonicated to disperse the pellet.

- 25 Ten μ l aliquots of this microprojectile-DNA mixture were placed on each macrocarrier and bombarded into maize suspension cells using a helium-driven microprojectile gun, PDS-1000 (1100 psi rupture disk pressure). Maintenance of embryogenic suspension cultures and transient expression assays were performed as previously described by Unger *et al.* (1993) *The Plant Cell* 5:831-841 and by

- 30 U.S. Patent No. 5,886,244.

Table 1

Treatment	EcR (μ g)	USP (μ g)	PHP11545 (μ g)
NosCLO only	PHP10956 (0.02)	none	5
NosCLO/NosUSP	PHP10956 (0.02)	PHP10697 (0.02)	5
Reporter only	none	none	5

Following cobombardment of the reporter and effector plasmids into the embryogenic suspension cells the transactivation of the Luciferase reporter construction was assayed. Transient transformed callus tissue was incubated on filter paper soaked with water or media and with ligand or the ethanol carrier. For the transient assays, 50 mg of callus tissue was incubated in the presence of ligand (10 μ M RH5992) or ethanol carrier control. The luciferase assays were performed as previously described (Unger *et al.* (1993) *The Plant Cell* 5:831-841) following 16 to 18 hours of incubation at 26°C.

Luciferase expression was measured in average light units and the results are summarized in Table 2. Expression of the USP receptor (plasmid PHP10967) and the C1::LexA::ecdysone fusion protein (plasmid PHP10956) transactivated the LexA responsive luciferase reporter (PHP11545) 53 fold in the presence of the RH5992 ligand (Table 2).

The USP receptor was not required for transactivation of the luciferase reporter. Expression of only the C1::LexA::ecdysone fusion protein (plasmid PHP10956) resulted in the transactivation of the LexA responsive luciferase reporter gene in the presence of the RH5992 ligand (Table 2).

Table 2

Effector(s)	Effector Dose (μ g)	RH5992	Average Light Units	Fold Induction
NosCLO only	0.02	None	18247	-
NosCLO only	0.02	10 μ M	153613	8.4x
NosCLO/NosUSP	0.02 each	None	3811	-
NosCLO/NosUSP	0.02 each	10 μ M	201808	53x
Reporter only	None	None	3843	-

Example 3:Transactivation of a Reporter Sequence by a Chimeric Ecdysone Receptor

To further examine the effect of the Ecdysone receptors on promoter-driven expression, a stable transactivation assay was performed using a chimeric Ecdysone receptor. DNA constructs were generated for the expression of both effector and reporter DNA sequences.

A reporter plasmid, designated PHP1654, was constructed in the pSP72 vector and contained 5 GAL4 responsive elements, a minimal CaMV-59 constitutive promoter, the Omega' leader sequence, Adh intron 1, the coding sequence for firefly Luciferase, and the PinII terminator sequences.

Two DNA constructions encoding chimeric Ecdysone receptor proteins were generated. The chimeric Ecdysone receptor construct designated PHP10512 contains the Nopaline synthase (NOS) promoter, the Omega' leader sequence, Adh1 intron, 355 promoter, PAT gene and the potato protease inhibitor II (PinII) termination sequences cloned into a pUC vector. A fragment encoding a fusion between the maize C1 transactivation domain, the Gal4 DNA binding domain, and the ligand-binding domain of the Ecdysone receptor was cloned downstream of the intron.

The plasmid designated PHP10513 also contains the Nopaline synthase (NOS) promoter, the Omega' leader sequence, Adh1 intron, 355 promoter, PAT gene, and the potato protease inhibitor II (PinII) termination sequences cloned into a pUC vector. However, the fragment encoding the chimeric receptor immediately downstream of the intron, comprises a fusion between the VP16 activation domain, the Gal4 DNA binding domain, and the ligand-binding domain of the Ecdysone receptor.

Immature embryos approximately 1.5 to 2.0 mm long from the Hi-II genotype (Armstrong and Phillips (1988) *Crop Sci.* 28:363-369) were bombarded with the PHP1654 reporter plasmid and either PHP10513 or PHP10512. As noted above, a selectable marker gene, PAT, was contained on PHP1050513 and PHP10512. The PAT gene confers resistance to the herbicide bialophos. (Wohlleben *et al.* (1988) *Gene* 70:25-37.)

Embryos were bombarded using the protocols described in Tomes *et al.* (1994) *Plant Cell, Tissue, and Organ culture, Fundamental Methods* 197-213. Specifically, the Hi-II immature embryos were bombarded and cultured on selection medium as described by Songstad *et al.* 1993 *Agronomy Abstracts* 183.

Plant regeneration from transgenic calli was performed according to the protocol of Armstrong and Phillips (1988) *Crop Sci* 28:363-369, except that bialaphos (3 mg/l) was added to each of the regeneration media. Plants were established in the greenhouse.

- 5 The transactivation of the luciferase reporter sequence by a chimeric Ecdysone receptor was assayed using standard protocols. Four leaf punches from each individual T₀ plant were placed on filter paper saturated with H₂O (w/EtOH carrier) or 10 μ M RH5992 in H₂O. Samples were incubated 16 hours and assayed for Luciferase activity. Results are shown in Light Units from both a negative
- 10 control (i.e. leaf punches saturated with H₂O) and RH5992-induced samples of 20 μ l of 200 μ l total extract assayed from individual plants. The results are shown in Table 3.

Table 3

Plasmid	Event Number	Sample Identification Number	Negative Control (Light Units)	RH5992 treated (Light Units)
PH10513	30	633295	140	37955
		633296	146	38507
		633297	153	37855
		633298	128	42346
		634477	129	38093
		634478	113	26394
		634479	136	24632
		634480	120	33636
		634481	129	28213
		634482	95	22137
		641482	505	105341
		642456	124	44639
		642457	348	49279
		642458	203	83241
		642459	609	61320
		642460	281	130618
		642461	324	95166
		645018	171	35641
PHP10512	78	645023	84	3771
		645024	86	1819
		645025	174	7742
		645026	154	1027
		645027	139	9742
		645028	120	2486
		645029	203	8494
		645030	75	3988
		645031	91	9265
		645032	242	6198
		645033	87	6293
		645034	101	1045
		645036	176	8354
		645037	84	4720
PHP10513	44	634930	17076	605147
		634931	45065	528950
		634932	15814	303021
		642462	36795	573842
		642463	7111	417072
		645019	11959	511220
		645020	15346	290095
PHP10513	117	652405	2122	90037
		652406	5711	40005
		652407	2988	149594
		652408	674	75379
		652409	2315	49243
		652410	3201	114913
		652411	203	57607
		652412	729	46075

The results of the stable transactivation assays show that expression of a chimeric Ecdysone receptor comprising the DNA binding domain of GAL4, the ligand binding domain of the Ecdysone receptor, and either the transactivation
5 domain from Maize C1 or VP16 can activate transcription of the appropriate target sequences in the presence of an Ecdysone receptor ligand.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All
10 publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be
15 obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED

1. An isolated nucleic acid sequence that encodes an insect protein from a *Pyrilidae* species, wherein said nucleotide sequence is selected from the group consisting of:
- 5 a) a nucleotide sequence comprising a sequence encoding an ecdysone receptor or Ultraspiracle;
- b) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1 or 3;
- 10 c) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2 or 4;
- d) a nucleotide sequence that hybridizes under stringent conditions to a sequence of a), b), or c).
- 15 2. An isolated polypeptide from a *Pyrilidae* species, wherein said polypeptide is selected from the group consisting of:
- a) a polypeptide sequence comprising an Ecdysone receptor or Ultraspiracle;
- b) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 2 or 4;
- 20 c) a polypeptide encoded by a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1 or 3;
- d) a polypeptide encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1 or 3.
- 25 3. A method of selectively inducing gene expression of a protein of interest in a plant, said method comprising:
- a) stably incorporating into the genome of said plant an expression cassette, said expression cassette comprising a promoter operably linked to a nucleotide sequence encoding an Ecdysone receptor, wherein said nucleotide sequence encoding the Ecdysone receptor is selected from the group consisting of:
- 30

- i) a nucleotide sequence from a *Pyralidae* species comprising a sequence encoding an Ecdysone receptor;
 - ii) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1;
 - 5 iii) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2;
 - iv) a nucleotide sequence that hybridizes under stringent conditions to a sequence of i), ii), or iii);
 - b) further stably incorporating into the genome of said plant a
10 second expression cassette, wherein said expression cassette comprises a transcriptional regulatory region operably linked to a nucleotide sequence encoding said protein of interest, and wherein said transcriptional regulatory region is activated by the ligand-receptor complex;
 - c) contacting said plant with a ligand which complexes with
15 said receptor, wherein said receptor-ligand complex interacts with the transcription regulatory region and induces gene expression of the protein of interest.
4. The method of claim 3, wherein said promoter is constitutive.
 - 20 5. The method of claim 3, wherein said promoter is tissue-preferred.
 6. The method of claim 3, wherein said tissue-preferred promoter is selected from the group consisting of anther-preferred, tapetal-preferred, ovule-preferred and pistil-preferred promoters.
 - 25 7. The method of claim 3, wherein said ligand is selected from the group comprising a non-steroidal ecdysteroid agonist, 20-hydroxyecdysone or an analog thereof.
 - 30 8. The method of claim 3, wherein expression of said protein of interest disrupts plant fertility.

9. The method of claim 3, wherein expression of said protein of interest induces fertility in an otherwise sterile plant.
10. The method of claim 3, wherein expression of said protein of interest induces an altered mode of reproduction.
11. The method of claim 10, wherein said altered mode of reproduction is apomixis.
12. The method of claim 3, wherein said Ecdysone receptor is a chimeric receptor, said chimeric receptor comprising an activation domain, a DNA binding domain, and a ligand binding domain, wherein at least one of the domains is from the Ecdysone receptor.
13. The method of claim 13, wherein the activation domain or the DNA binding domain is from a transcriptional activator protein.
14. The method of claim 10, wherein the transcriptional activator protein is selected from the group comprising VP16, maize C1, or GAL4.
15. The method of claim 12, wherein the activation domain, the DNA binding domain or the ligand-binding domain is from a member of the steroid/thyroid hormone superfamily of nuclear receptors.
16. The method of claim 15, wherein the member of the steroid/thyroid hormone superfamily of nuclear receptors is selected from the group comprising retinoic acid receptor, vitamin D receptor, or thyroid hormone receptor.
17. The method of claim 3, wherein said expression cassette further comprises a nucleotide sequence encoding a promoter operably linked to a nucleotide sequence encoding Ultraspiracle, and said nucleotide sequence encoding Ultraspiracle is selected from the group consisting of:

- a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 3;
- b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 4;
- 5 c) a nucleotide sequence that hybridizes under stringent conditions to a sequence of a) or b).

18. The method of claim 17, wherein said Ultraspiracle receptor is a chimeric receptor, said chimeric receptor comprising an activation domain, a DNA
10 binding domain, and a ligand binding domain, wherein at least one of the domains is from the Ultraspiracle receptor.

19. The method of claim 17, wherein the nucleotide sequences encoding the Ecdysone receptor and Ultraspiracle are contained on separate
15 expression cassettes.

20. The method of claim 3, wherein said plant is a dicot.

21. The method of claim 3, wherein said plant is a monocot.
20

22. The method of claim 18, wherein said monocot is maize.

23. An expression vector comprising a promoter operably linked to a nucleotide sequence encoding an Ecdysone receptor wherein said nucleotide
25 sequence encoding the Ecdysone receptor is selected from the group consisting of:

- a) a nucleotide sequence from a *Pyrilidae* species comprising a sequence encoding an Ecdysone receptor;
- b) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1;
- 30 c) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2;
- d) a nucleotide sequence that hybridizes under stringent conditions to a sequence of a), b), or c).

24. The expression vector of claim 20, wherein said Ecdysone receptor is a chimeric receptor, said chimeric receptor comprising an activation domain, a DNA binding domain and a ligand binding domain, wherein at least one of the domains is from the Ecdysone receptor.

25. An expression vector comprising a promoter operably linked to a nucleotide sequence encoding Ultraspiracle, and said nucleotide sequence encoding Ultraspiracle is selected from the group consisting of:

- 10 a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 3;
- b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 4;
- c) a nucleotide sequence that hybridizes under stringent
- 15 conditions to a sequence of a) or b).

26. A plant having stably incorporated in its genome the expression vector of claim 23.

20 27. The plant of claim 26, wherein said plant further comprises a second expression cassette stably incorporated in its genome, said second expression cassette comprising a transcriptional regulatory region operably linked to a nucleotide sequence encoding a protein of interest, wherein said transcriptional regulatory region is activated by a ligand-receptor complex.

25

28. The plant of claim 27, wherein said plant further comprises the expression vector of claim 25 stably incorporated in its genome.

29. The seed of the plant of claim 26.

30

30. The seed of the plant of claim 27.

31. The seed of the plant of claim 28.

32. A plant cell having stably incorporated in its genome the expression vector of claim 23.

5 33. The plant cell of claim 32, wherein said plant cell further comprises a second expression cassette stably incorporated in its genome, said second expression cassette comprising a transcriptional regulatory region operably linked to a nucleotide sequence encoding a protein of interest, wherein said
10 transcriptional regulatory region is activated by a ligand-receptor complex.

34. The plant cell of claim 33, wherein said plant cell further comprises the expression vector of claim 25 stably incorporated in its genome.

SEQUENCE LISTING

<110> Albertsen, Marc C.
 Brooke, Catherine D.
 Garnaat, Carl W.
 Roth, Bradley A.

<120> NOVEL ECDYSONE RECEPTORS AND METHODS FOR THEIR USE

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/20715

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/82 C12N5/10 C07K14/72 A01H5/00
A01H5/10 C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 27673 A (CIBA GEIGY AG) 12 September 1996 (1996-09-12) cited in the application the whole document ---	1-34
X	WO 96 37609 A (ZENECA LTD ;JEPSON IAN (GB); MARTINEZ ALBERTO (GB); GREENLAND ANDR) 28 November 1996 (1996-11-28) cited in the application abstract; claims 1-95; figures 6,36-38; examples 1-9 page 1 -page 13 ----- -/--	1-4,7, 12-15, 20-24, 26,27, 29,30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search

23 February 2000

Date of mailing of the international search report

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/20715

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